

# Functions of CD40 on B cells, dendritic cells and other cells

Cees van Kooten\* and Jacques Banchereau†

CD40 is a cell surface receptor that belongs to the tumor necrosis factor receptor family. It was first identified and functionally characterized on B lymphocytes; however, in recent years it has become clear that CD40 expression is much broader, as it is found on monocytes, dendritic cells, hematopoietic progenitors, endothelial cells and epithelial cells. Although initially identified for its activation properties, CD40 is also able to transduce negative signals in various cell types. It is presently accepted that CD40 plays a critical role in the regulation of immune responses. The past year has seen considerable progress in the identification of intracellular molecules mediating CD40 signaling. Furthermore, it has been established that ligation of CD40 ligand (CD40L) delivers signals to the CD40L bearing cells themselves. Finally, the critical role of CD40-CD40L interactions in the development of various disease states has been fully appreciated.

## Addresses

\*Department of Nephrology, Leiden University Hospital, 1-C3P, Albinusdreef 2, 2333 AA Leiden, The Netherlands;  
e-mail: Kooten@ruff2.medfac.leidenuniv.nl  
†Baylor Institute for Immunology Research, 3500 Gaston Avenue, 2nd Floor Hobitelle, Dallas, TX 75246, USA;  
e-mail: j.banchereau@baylorirdallas.edu  
Correspondence: J Banchereau

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## Abbreviations

APC antigen-presenting cell  
EBV Epstein-Barr virus  
GM-CSF granulocyte-macrophage colony-stimulating factor  
IL interleukin  
L ligand  
Th T helper  
TNF tumor necrosis factor  
TNFR TNF receptor  
TRAF TRAF-associated factor

## Introduction

Twelve years after the identification of the CD40 antigen using monoclonal antibodies, a wealth of information has been generated which identifies CD40 and its ligand (L) as critical entities in the regulation of immune responses (reviewed in [1-5]). Early studies, which concentrated on the role of CD40 in B-cell physiology, culminated with the finding that a defective CD40-CD40L interaction (generated by mutations in the CD40L gene) is actually the cause of the X-linked immunodeficiency hyper-IgM syndrome. Since this finding, the availability of specific molecular tools and the generation of both CD40 and CD40L knockout mice have extended the research in

much broader ways. Recent investigations have resulted in an explosion of data concerning CD40 and CD40L: the structure and the expression of CD40 and its ligand; the signal transduction mechanisms of CD40; the functional expression of CD40 on cells other than B cells; and the *in vivo* role of CD40-CD40L interactions. These points will be discussed in this review. Because of space limitations, citations will refer to the most recent publications and for further information, the reader is referred to several concise reviews which have been published recently [1-5].

## Structure and expression of CD40 and its ligand

CD40-CD40L (CD154) belongs to the emerging receptor-ligand families composed of type I-type II transmembrane proteins respectively. Receptor-ligand pairs which belong to these families include tumor necrosis factor (TNF) and its receptor (TNFR), CD27-CD70, CD30-CD30L, and Fas(CD95)-FasL [6]. In the past year several new members of the TNFR-TNF gene families have been cloned. The families are characterized by clustered chromosome locations and their products by structural homologies and overlapping biological activities in processes such as cell growth, differentiation and death. Distribution of the molecules ranges from very broad to restricted. For example, CD40, initially seen as a B-cell-specific receptor, is now recognized as being fairly widely distributed (Table 1). In contrast, CD40L is mainly expressed by activated CD4<sup>+</sup> T cells. Recently, however, CD40L expression on basophils, eosinophils, activated B cells and blood dendritic cells has also been reported [7-9].

## CD40 signal transduction

Studies on CD40 signal transduction have now yielded several mediators and pathways involved in this cellular activation mechanism [10]. It should be noted, however, that most of the studies have been performed with B cells and that alternative pathways may very well be operational in other cell types. Like all other members of the TNFR family, CD40 has no kinase domain and no known consensus sequence for binding kinases. Yet, CD40 ligation activates protein tyrosine kinases, including Lyn and Syk, and induces the tyrosine phosphorylation of multiple substrates, including phosphatidylinositol 3-kinase and phospholipase C- $\gamma$ . PLC- $\gamma$  is activated by phosphorylation. This phosphorylation results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PI 4,5-P<sub>2</sub>) and, thereby, the generation of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP<sub>3</sub>). Such increases in IP<sub>3</sub> have indeed been shown using CD40 antibodies to stimulate B cells (reviewed in [3]). Interestingly, CD40 ligation also appears to activate

**Table 1****Cells expressing CD40 and functional consequences of *in vitro* CD40 activation.**

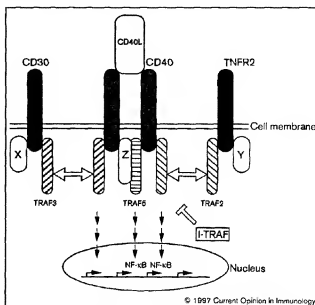
Cell type	Functional consequences
Pre-B cells	Proliferation; CD23 expression
Naive mature B cells	Proliferation, differentiation isotype switch
Germinal center B cells	Proliferation, differentiation Fas expression, B-cell selection
Plasma cells	IL-6 production
T cells	Proliferation, CD25 expression Cytokine production
Monocytes	Cytokine secretion NO production Production of metalloproteinases Monocyte procoagulant activity
Dendritic cells	Growth and survival Expression of co-stimulatory molecules Enhanced cytokine production
CD34 <sup>+</sup> precursors	Proliferation Development of dendritic cells
Eosinophils	Enhanced survival GM-CSF production
Endothelial cells	Upregulation of CD54, CD62E, CD106
Thymic epithelial cells	GM-CSF production
Kidney epithelial cells	Cytokine/chemokine secretion: IL-6, IL-8, MCP-1, RANTES
Keratinoocytes	Enhanced expression of CD54, Bcl-x IL8 secretion
Carcinomas	Growth inhibition
Fibroblasts/synoviocytes	Proliferation; cytokine production
Follicular dendritic cells	Growth; CD54 expression

MCP-1, macrophage chemoattractant protein-1; NO, nitric oxide; RANTES, regulated on activation, normal T cell expressed and secreted.

the serine/threonine protein kinases and in particular the stress-activated protein kinases (SAPKs, also known as JNK for c-Jun amino-terminal kinase) and other members of the mitogen-activated protein (MAP) kinase family [11–15]. Finally, all the above different activation pathways result in the activation of various transcription factors, including nuclear factor (NF)- $\kappa$ B, NF- $\kappa$ B-like molecules (such as p50, p65 [rel A], cRel), c-Jun and nuclear factor of activated T cells (NFAT).

In recent years it has been established that members of the TNFR family associate intracellularly with different families of signaling molecules, including the

'death domain' family and the TNFR-associated factor (TRAF) family [16]. Using the two-hybrid system, such protein-protein interactions have been demonstrated for TNFR1, TNFR2, Fas, CD30 and CD40. Interestingly, although there is no cross-reactivity between the extracellular ligands of the TNFR family (with the exception of TNF and lymphotoxin [LT]), the intracellular ligands seem to be much more promiscuous and form a complex network of homodimers and heterodimers (Fig. 1).

**Figure 1**

Schematic representation of the molecules involved in CD40 signal transduction. X, Y and Z (indicated by the open structures) represent as yet unidentified molecules associated with the signaling complexes of TNFR members. TRAF2 and TRAF3, which are indicated by hatched figures, associate with CD40, but are also shared by other members of TNFR family (namely TNFR2 and CD30, respectively) as indicated by the double-headed arrows. TRAF6 is a recently identified member of the TRAF family which can also induce NF- $\kappa$ B activation. Activation processes are initiated when the CD40 receptor becomes cross-linked by interaction with CD40L. Intracellular association of TRAF2 and I-TRAF results in inhibition of TRAF2-mediated signal transduction. Thin vertical arrows represent signal transduction pathways leading to NF- $\kappa$ B activation. Right angled arrows represent transcription induced by active NF- $\kappa$ B. For more details, readers are referred to the text.

CD40 interacts with TRAF3, also identified under the name CD40 receptor associated factor 1 (CRAF1), CD40 binding protein (CD40bp), LMP-1 associated protein 1 (LAP1) and CD40 associated protein 1 (CAP1) [17\*,18,19\*,20\*]. TRAF3 is a 62 kDa intracellular protein which is expressed in almost all cell types. The protein contains several functional domains including a zinc finger and a RING finger which may possibly be involved in DNA binding, as well as a TRAF domain which may function in protein-protein interactions. TRAF3 is suggested to be

important for CD40 signaling inasmuch as a functionally inactive mutant of CD40 (Thr234→Ala) does not bind TRAF3 [18]. TRAF3 has been demonstrated to associate with CD30, although the functional consequence of this interaction remains unknown. Interestingly, TRAF3 also binds to the Epstein-Barr virus (EBV) transforming gene product, latent infection membrane protein 1 (LMP1), which indicates that EBV may utilize the CD40 signaling pathway to activate and immortalize B lymphocytes [19,21]. The unaffected activation signals of B cells from TRAF3<sup>-/-</sup> mice suggests a dispensable role for TRAF3 in B cells which contrasts with the altered signals observed in TRAF3<sup>-/-</sup> T cells [22\*].

In addition, CD40 has been demonstrated to associate with TRAF2, a molecule which also associates with TNFR2. The induction of NF- $\kappa$ B activation after cross-linking of CD40 via CD40L, and also after TNFR2 cross-linking via TNF, could be attributed to TRAF2 signaling, as a truncated TRAF2 protein lacking the amino-terminal RING finger domain mediated dominant-negative inhibition of TRAF2 signaling [23\*]. Furthermore, TRAF2-mediated NF- $\kappa$ B activation following either CD40 or TNF stimulation can be prevented by an inhibitor protein called I-TRAF [24\*]. Finally, the gene encoding a new CD40-associated protein, termed TRAF5, was recently cloned [25\*]. TRAF5, which does not bind to TNFR2, seems to be associated with the same cytoplasmic region of CD40 (amino acid residues 230–269) as observed for TRAF3. Over-expression of TRAF5 results in the activation of NF- $\kappa$ B, but it is not known whether this is sensitive to I-TRAF.

## Functions of CD40 *In vitro*

### B lymphocytes

CD40-activated B cells enter into a proliferative state which is further stimulated by addition of cytokines such as interleukin (IL)-4, IL-13 or IL-10 and their combination [26]. Cytokines can also induce CD40-activated B cells to secrete immunoglobulins (Igs), with IL-10 inducing the secretion of large quantities of Igs as a consequence of inducing plasma-cell differentiation. In addition, IL-10 also acts as a factor inducing a switch towards IgG<sub>1</sub> and IgG<sub>3</sub> production, as demonstrated at the molecular level by the appearance of 'switch circles', the non-replicating excision DNA which is formed as a consequence of isotype switching [27\*]. A combination of IL-10 and transforming growth factor (TGF)  $\beta$  induces IgA production in CD40-activated B cells, and either IL-4 or IL-13 induces CD40-activated B cells to switch towards IgG<sub>4</sub> and IgE production.

CD40 ligation activates resting B cells as shown by the increase in B cell size and the increased level of expression of cell surface molecules involved in homotypic and heterotypic aggregation (CD23, very late antigen [VLA]-4) as well as by T-cell co-stimulation (CD80 or CD86).

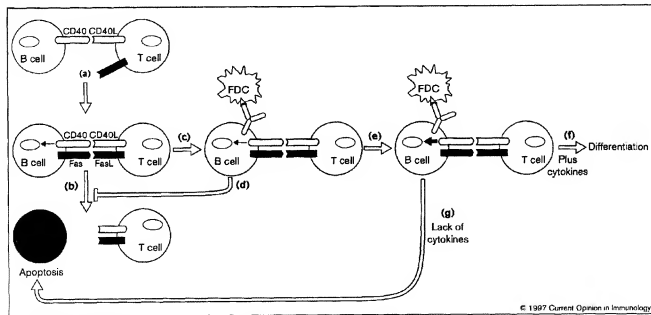
Interestingly, CD40 activation of B cells also results in the induction of Fas expression, and renders cells susceptible to Fas-induced apoptosis [28,29]. In fact, together with B cell antigen receptor cross-linking, these three receptors (Fas, CD40 and the B cell antigen receptor) generate a complex network of positive and negative signals whereby the response of the B cell, activation or death (Fig. 2), is determined by its differentiation stage [30\*,31–33]. Importantly, dual triggering of resting B lymphocytes through their CD40 and antigen receptors induces a phenotype characteristic of cells from germinal centers (CD38\*, CD95\*, carboxypeptidase\*, CD71\*, CD86\*, but CD24-). Germinal centers constitute the anatomical site where B cells undergo somatic mutation, selection, and isotype switching and where they become either plasma or memory cells [34,35]. Prolonged triggering of CD40 skews the maturation of B cells into memory cells whereas interruption of CD40 signaling allows plasma-cell differentiation [36\*,37,38]. Although most normal and malignant B cells proliferate in response to CD40 engagement, plasma cells appear unresponsive [39].

### Monocytes and dendritic cells

The idea that CD40 is expressed on professional antigen-presenting cells (APCs) like monocytes and dendritic cells is now well established [4\*]. Low spontaneous expression of CD40 can be detected on freshly isolated monocytes, and can be upregulated by cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3 and interferon (IFN)- $\gamma$ . In contrast, CD40 is expressed at high levels on dendritic cells isolated from different tissues or generated *in vitro* by culturing hematopoietic progenitors. Interestingly, CD40 expression has also been detected on monocytes infiltrating brain lesions of patients with multiple sclerosis [40\*], indicating that these cells have been in contact with cytokines such as IFN- $\gamma$  or GM-CSF.

CD40 ligation of monocytes and dendritic cells results in the secretion of multiple proteins including cytokines such as IL-1, IL-6, IL-8, IL-10, IL-12, TNF $\alpha$ , macrophage inhibitory protein (MIP) 1 $\alpha$ , as well as enzymes such as matrix metalloproteinase (MMP) [41]. Importantly, the secretion of IL-12 allows a skewing of T-cell maturation towards the T helper (Th) 1 pathway [4\*,5\*]. In addition, CD40 ligation considerably alters the phenotype of these APCs by upregulating the expression of co-stimulatory molecules such as CD54 (intercellular adhesion molecule [ICAM]-1), CD58 (lymphocyte function-associated antigen [LFA]-3), CD80 (B7-1), CD86 (B7-2) (Fig. 3). Interrupting CD40–CD40L interactions in T cell and dendritic cell co-cultures results in reduced T-cell proliferation, possibly as a consequence of both altered CD40 signaling to the APCs (leading to reduced expression of co-stimulatory membrane molecules and cytokines) and altered CD40L signaling to the T cells. Although *in vitro* results were initially described, these results are now being confirmed *in vivo* [42\*,43\*].

Figure 2



Interactions between CD40, Fas and BCR signaling determine the outcome of B cell stimulation: activation or death. (a) Cross-linking of CD40 on B cells by CD40L on activated T cells results in B cell activation (indicated by the left facing arrow within the B cell), as well as the induction of Fas expression on the B cell surface. (b) Subsequent Fas ligation with FasL on activated T cells induces apoptosis of B cells thereby terminating the immune response. (c) When the BCR (represented by the Y shaped structure) is triggered simultaneously with CD40 and Fas, as probably takes place in germinal centers (where unprocessed antigen, represented by the triangular structure, is presented by follicular dendritic cells [FDC]), Fas-induced apoptosis is inhibited (d) whereas (e) CD40 activation of B cells is augmented (indicated by the thick left facing arrow). (f) This increased activation, in the presence of cytokines, results in B cell differentiation. (g) Note, however, that prolonged triggering of CD40 and BCR in the absence of other T cell signals (such as cytokines) results in the deletion of B cells (possibly reflecting what happens during negative selection of autoreactive B cells).

Ligation of CD40 enhances the survival of dendritic cells and monocytes. Of note, CD40 ligation of CD34<sup>+</sup> hematopoietic progenitors induces their proliferation and differentiation into cells with prominent dendritic cell attributes [44<sup>\*</sup>]. The importance of this receptor-ligand pair for the cellular immune response has been demonstrated by the diminished immunity of CD40 and CD40L knockout mice against several pathogens [5<sup>\*</sup>]. In keeping with this, CD40 ligation activates monocyte tumoricidal activity as well as nitric oxide synthesis.

#### Endothelial cells, epithelial cells and fibroblasts

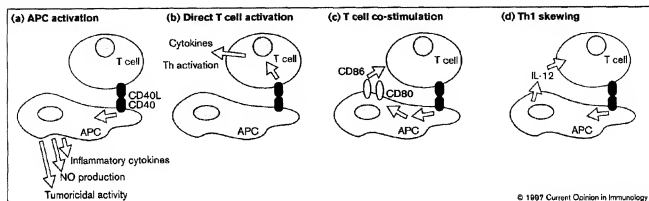
Immunohistology performed on various tissue sections has shown that anti-CD40 antibodies stain vascular endothelium, epidermal basal membrane, scattered fibroblasts, thymic epithelium and follicular dendritic cells (for specific references see reviews [1<sup>\*</sup>-5<sup>\*</sup>]). Recently, CD40 expression was demonstrated in lesions of Kaposi's sarcoma, as well as on vascular endothelium in areas adjacent to the tumors [45]. Consistently, primary lines of endothelial cells, thymic and kidney tubular epithelial cells, keratinocytes, skin and synovial fibroblasts as well as follicular dendritic cells express CD40. These adherent cells express a functional CD40 whose ligation induces phenotypic alterations and cytokine secretion as well as

stimulation or inhibition of proliferation (Table 1). In particular, endothelial cells display marked upregulation of CD54 (ICAM-1), CD106 (vascular cell adhesion molecule [VCAM]-1) and CD62E (E-selectin), resulting in increased ability to bind leukocytes. Furthermore, cross-linking of CD40 on keratinocytes results in the inhibition of proliferation and a subsequent induction of differentiation [46].

#### *In vivo* functions of CD40-CD40L interactions Hyper-IgM syndrome

The first demonstration of the critical role of CD40-CD40L interactions *in vivo* came from the discovery that the hyper-IgM syndrome, an X-linked immunodeficiency, is due to a genetic alteration of CD40L [47]. The disease is characterized by a severe impairment of T-cell-dependent antibody responses and a lack of memory B cells and circulating IgG, IgA and IgE. Patients with hyper-IgM syndrome have an enhanced susceptibility to opportunistic infections, such as *Pneumocystis carinii* pneumonia and *Cryptosporidium* diarrhea. The disease characteristics indicate a role for CD40-CD40L interactions in cell-mediated immune responses. Indeed, CD40L knockout mice display a considerable impairment of antigen specific T-cell priming and appear particularly susceptible to

Figure 3



Functional consequences of CD40-CD40L interactions between activated T cells and APCs (monocytes and dendritic cells). The functional consequences of CD40 cross-linking on B cells are not included in this figure. Although most pathways are operational at the same time, for reasons of clarity processes are separated into: (a) APC activation, (b) direct T cell activation, (c) T cell co-stimulation, and (d) Th1 skewing. (a) APC activation via CD40 results in the production of inflammatory cytokines, nitric oxide (NO) and an increase in tumoricidal activity. (b) Interaction between CD40 and CD40L can also result in signaling via CD40L and result in T cell cytokine production and the generation of helper activity. (c) Activation of APC via CD40 results in a strong increase in CD80 and CD86 expression. Both co-stimulatory molecules are important for an efficient and 'productive' T cell activation. (d) Activation of APC via CD40 also results in IL-12 production, which has a major influence on the balance of the development of Th1 type T cells.

*Leishmania* infection [48\*-50\*]. This most likely results from a defective Th1 response which is related to an impaired production of IL-12 by APCs.

#### Formation of germinal centers

Germinal centers are the anatomical sites in lymphoid organs where isotype switching and somatic mutations are initiated. In one study [51], it was demonstrated that patients with the hyper-IgM syndrome lack germinal centers in their lymphoid organs. In accordance, somatic mutations within variable (V), diversity (D) and joining (J) VDJ transcripts could not be detected in the circulating B cells from five out of six patients [52,53]. Interestingly, the patient who had almost normal levels of somatic mutations, displayed a mutation within the transmembrane region of CD40L which resulted in a very transient CD40L expression on activated T cells [53]. These data suggest that a minimal level of CD40L expression is sufficient to allow the activation of events leading to the introduction of somatic mutations *in vivo*. CD40 activation of B cells *per se*, however, is not sufficient to induce somatic mutations *in vitro*, as it requires activated T cells.

Studies with a CD40-Fc fusion protein have highlighted the important role of T-cell signaling through CD40L in the development of Th function. In particular, administration of soluble CD40-Fc *in vivo* to CD40 knockout mice initiates germinal center formation. Furthermore, T cells primed in the absence of CD40 are unable to help normal B cells to class switch Ig and to form germinal centers [54\*]. Regarding this role of T cells, it has been shown that ligating the CD40L of human activated T cells, by specific monoclonal antibodies or by using

CD40 transfected T cells, considerably enhances their production of cytokines [55\*,56\*].

#### CD40-CD40L: role in infection and immunity

The availability of blocking anti-mouse-CD40L antibodies and both CD40 and CD40L knockout mice has offered the possibility to test the role of CD40-CD40L interactions in several disease models. These include models of autoimmunity, infection with micro-organisms and transplantation tolerance (Table 2).

Administration of antibodies to CD40L has been shown to prevent the establishment of autoimmune symptoms in various murine models including those for collagen type II induced arthritis (a model of human rheumatoid arthritis) [57], lupus nephritis in lupus-prone mice (which represent models of systemic lupus erythematosus) [58], and proteolipoprotein induced experimental encephalomyelitis (a model of human multiple sclerosis) [40\*]. Importantly, in the last case, the antibody could induce an important reduction of the disease even when administered after disease onset [40\*].

With the knowledge that CD40 expression can be found in every arm of the immune system (B cells, T cells, monocytes), it does not come as a surprise that blocking this activation pathway has negative effects on the immune defense against pathogens such as *Leishmania* [48\*-50\*]. Only in some cases of antiviral responses (lymphocytic choriomeningitis virus, pichinde virus and vesicular stomatitis virus) was normal immunity observed in CD40L knockout mice. In addition, it appeared that protective antibodies against *Borrelia* infection developed normally in CD40L-deficient mice [59].

Table 2

*In vivo* role of CD40-CD40L in animal models with (a) anti-CD40L treatment and (b) CD40 and/or CD40L knockout mice.

Model	Effect
<b>(a)</b>	
Collagen arthritis	Diminished joint inflammation; lower serum Ab levels; low level infiltration
Lupus nephritis	Delayed disease onset with reduced disease incidence
Graft-versus-host disease	Inhibits donor allospecific Th cells
Experimental allergic encephalomyelitis	Disease prevention and reduction of clinical signs
Transplantation	Increased survival of pancreatic islet allografts when treated in combination with allogeneic lymphocytes Long term acceptance of skin and cardiac allografts when treated in combination with CTLA4-Ig
Allergic contact dermatitis	Long lasting unresponsiveness when treated in combination with CTLA4-Ig
Pneumocystis pneumonia	Increased susceptibility to <i>Pneumocystis carinii</i> infection
<b>(b)</b>	
Leishmania infection	Reduced resistance to <i>Leishmania major</i> and <i>Leishmania amazonensis</i> infection; more severe lesions than wild type
Antiviral immunity	Infection with LCMV results in severely compromised antiviral Ab responses, normal primary CTL responses but reduced memory CTL response
Experimental allergic encephalomyelitis	No disease development because of defective T-cell function and IFN $\gamma$ production
Murine Lyme disease	Normal protective Abs against <i>Borrelia burgdorferi</i>

Ab, antibody; CTL, cytotoxic T lymphocyte; CTLA, CTL antigen; LCMV, lymphocytic choriomeningitis virus.

Administration of anti-CD40L antibodies prevents the development of graft-versus-host disease (GVHD) that occurs as a major complication of allogeneic bone marrow transplantation [60,61]. Furthermore, a combination of allogeneic B cells and anti-CD40L antibody considerably decreases host reactivity of both CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes, thereby allowing efficient transplantation of allogeneic pancreatic  $\beta$  islet cells [62]. Anti-CD40L antibodies markedly extend the survival of cardiac allografts in both naive and sensitized hosts when administered at the time of transplantation [63]. More importantly, even long term acceptance of skin and cardiac allografts can be obtained by a simultaneous blocking of the CD40 and CD28 pathways [64\*\*].

## Conclusions

In recent years, the focus of CD40 research has been shifted away from the study of B-cell regulation (humoral immunity) towards the study of CD40 as a general regulator of immune and inflammatory processes. In particular, the finding of CD40 expression on activated endothelium has important clinical implications, as it places this molecule in the centre of (chronic) inflam-

mation, transplantation, tumor metastasis, angiogenesis and normal leukocyte trafficking. Furthermore, multiple disease states appear to be improved by the interruption of CD40-CD40L interactions. This is presently accomplished by preventing the association of the receptor with its ligand using specific antibodies or soluble receptor molecules. For clinical applications, however, such reagents may not prove to be useful therapeutic entities. The identification of small synthetic chemical agents which prevent the interaction of CD40 with its ligand would be of interest. Alternatively, by unravelling the signal transduction pathways of CD40 in different target cells, pharmacological agents may be developed which will specifically block the intracellular pathways turned on after ligation of either CD40 or CD40L. The recent identification of a dominant-negative inhibitor of CD40 activation might represent a first step in this direction.

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